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Inhibition of Aflatoxin B_1 Production by *Aspergillus parasiticus* Using Nonaflatoxigenic Strains: Role of Vegetative Compatibility

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The effect of vegetative compatibility on the inhibition of aflatoxin B_1 production by *Aspergillus parasiticus* was examined using nonaflatoxigenic strains. Nonaflatoxigenic white-conidial mutants were paired in different proportions on an agar medium with aflatoxigenic yellow-conidial mutants belonging to the same isolate, to the same vegetative compatibility group but with the original wild types differing in phenotype, and to different vegetative compatibility groups. Heterokaryosis as a result of hyphal anastomosis was detected by the presence of conidiogenous structures with a mixture of green and parental (white and/or yellow) chains of conidia. Sclerotium production (number and dry weight) was significantly greater in pairings of compatible strains that formed heterokaryons than in pairings of strains from different vegetative compatibility groups. In contrast, there were no consistent differences in aflatoxin B_1 inhibition by nonaflatoxigenic strains in pairings from the same vegetative compatibility group and pairings from different groups. Therefore, the composition of vegetative compatibility groups within a population may be of minor importance in predicting the efficacy of a particular nonaflatoxigenic strain for the biological control of aflatoxin contamination of crops.

Key Words: **aflatoxin; *Aspergillus parasiticus;* biological control; intraspecific competition; vegetative compatibility.**

INTRODUCTION

Crops such as corn, cottonseed, and peanuts are frequently invaded by *Aspergillus flavus* Link and *A. parasiticus* Speare, fungi that produce the carcinogenic aflatoxins in agricultural commodities. Drought stress and elevated temperatures during seed maturation increase the susceptibility of these crops to fungal invasion and subsequent contamination with aflatoxins (Hill *et al.*, 1983; Jones *et al.*, 1981; Klich, 1987). *A. flavus* is typically the dominant aflatoxigenic species (Schroeder and Boller, 1973), although *A. parasiticus* can contribute to aflatoxin contamination to varying degrees, particularly in peanuts (Hill *et al.*, 1985). The two species also differ in the types of aflatoxins produced. *A. flavus* produces aflatoxins B_1 and B_2, and isolates vary considerably in their genetic capacity to produce aflatoxins, with many being nonaflatoxigenic (Horn and Dorner, 1999). In contrast, isolates of *A. parasiticus* produce aflatoxins G_1 and G_2 in addition to the B aflatoxins, and nonaflatoxigenic isolates of *A. parasiticus* rarely have been reported from nature (Horn *et al.*, 1996). Of the four aflatoxins, B_1 is the most toxic to animals (Cullen and Newberne, 1994).

In *Aspergillus* species, stable hyphal anastomosis between individuals of different genotypes is a prerequisite for heterokaryosis and is genetically controlled by a series of *het* loci in which the occurrence of different alleles at one or more loci results in incompatibility (Leslie, 1993). Populations of *Aspergillus* species can thus be examined as subpopulations of vegetatively compatible individuals. In *A. flavus* and *A. parasiticus*, populations are extremely diverse genetically and comprise large numbers of vegetative compatibility groups (VCGs), even within a restricted geographic area (Bayman and Cotty, 1991; Horn and Greene, 1995; Papa, 1986).

Biological control has been used to reduce aflatoxin contamination in various crops (Cotty, 1994; Dorner *et al.*, 1998). This technique involves the application to field soil of a nonaflatoxigenic strain of *A. flavus* and/or *A. parasiticus*, resulting in a high population density that allows the biocontrol strain to effectively compete with native aflatoxigenic strains during invasion under conditions favorable for aflatoxin contamination. Invasion of a seed solely by the biocontrol strain would be expected because of its high density relative to wild-type strains in the field. However, the high density of the biocontrol strain also suggests that when a seed is invaded by a native aflatoxigenic strain, the native strain may be competing with the biocontrol strain for

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the substrate. Even in wild populations of *A. flavus*, infection of seeds from single cotton boll locules and from individual peanut pods often involves multiple isolates belonging to different VCGs (Bayman and Cotty, 1991; Horn and Greene, 1995).

Although the effect of interspecific competition on aflatoxin production by *A. flavus* and *A. parasiticus* has been extensively studied (Nout, 1989; Ramakrishna *et al.*, 1996; Wicklow *et al.*, 1980), less attention has been given to the role of intraspecific competition in aflatoxin contamination. Intraspecific competition is the basis for biological control of aflatoxins, since the introduced nonaflatoxigenic strain presumably occupies the same ecological niche as native aflatoxigenic strains and, as a consequence, effectively competes with them. Rayner (1991) postulated that within a species, interactions between vegetatively compatible individuals are more cooperative due to hyphal anastomoses than interactions between incompatible individuals. Therefore, a biological control strain may not inhibit aflatoxin production with strains from the same VCG as effectively as with strains from different VCGs. To test this hypothesis, the inhibitory effect of nonaflatoxigenic mutants of *A. parasiticus* on aflatoxin production was examined in culture when paired with aflatoxigenic strains belonging to (1) the same isolate, (2) the same VCG but with the original wild types differing in phenotype, and (3) different VCGs.

MATERIALS AND METHODS

Aspergillus parasiticus *isolates and mutations.* Pairs of wild-type isolates of *A. parasiticus* representing VCGs 2, 3, 5, and 9 were obtained from soil in a single peanut field (Horn and Greene, 1995). Within each VCG, isolates that differed phenotypically were chosen from widely separated areas of the field. Isolates were characterized by their production of mycotoxins, by the number of sclerotia per plate, and by sclerotium shape (length/width ratio) (Table 1). Aflatoxin and kojic acid production in vials containing an enriched liquid medium were quantified using high-performance liquid chromatography (HPLC) and sclerotia produced on center-point-inoculated plates were measured with a stereomicroscope according to the methods of Horn *et al.* (1996).

Designations for conidial-color mutations of isolates are *w* = white and *y* = yellow, and mutations resulting in nondetectable levels of aflatoxins B_1, B_2, G_1, and G_2 in culture are designated *afl*$^-$. All *A. parasiticus* strains are maintained at the National Peanut Research Laboratory, Dawson, Georgia.

To obtain *w* and *y* mutants, wild-type isolates were grown on Czapek agar (Cz) slants for 7 days at 30°C. Conidia were scraped from slants in sterile water containing Tween 20 (100 µl/liter), and the suspension was filtered through glass wool and adjusted with water to 10^5 conidia/ml. Ten milliliters of conidial suspension was added to a 9-cm sterile glass petri dish bottom, and conidia were mutagenized under short-wavelength UV (254 nm) at 140 mJ/cm using a UV Crosslinker (Fisher Scientific, Pittsburgh, PA). After thoroughly stirring the suspension, 0.2-ml aliquots were spread on Cz plates that were then incubated for 7 days at 30°C.

White-conidial mutants were further exposed to UV as described above for producing *w afl*$^-$ mutants. Survivors on Cz plates that demonstrated wild-type characteristics of growth and amount of sporulation were transferred to slants of coconut cream agar (CCA) (Dyer and McCammon, 1994) consisting of 25% (w/v) cream of coconut (Coco López, Dominican Republic) and 1.5% agar. Slants were incubated in darkness for 7 days at 30°C and then examined under long-wavelength UV (365 nm) for fluorescence in the medium. For suspected *w afl*$^-$ mutants that did not fluoresce on CCA, dry conidia (approximately 10^5) were transferred to two replicate 4-ml vials containing 1 ml of modified yeast extract–sucrose (mYES) broth consisting of sucrose, 50 g; yeast extract, 5 g; and distilled water, 1 liter. The pH was adjusted to 6.5 with HCl. Vial cultures were incubated in darkness for 7 days at 30°C and then extracted and prepared for HPLC analysis as previously described (Horn *et al.*, 1996). Aflatoxins B_1, B_2, G_1, and G_2 were quantified by injecting 20 µl of prepared extract into an HPLC system consisting of an Acuflow Series III pump (Fisher Scientific), a Waters Model 717 autosampler, a photochemical reactor for fluorescence enhancement (Aura Industries, Staten Island, NY), a Shimadzu Model RF-551 fluorescence detector, and a Shimadzu Class VP chromatography laboratory automated software system. Aflatoxins were separated on a Waters Nova-PAK C_{18} reversed-phase column (3.9 mm ID × 150 mm; 4-µm particle size) with a mobile phase of water–methanol–butanol (70:35:0.6, v/v/v) at a flow rate of 0.8 ml/min. Calibration was achieved with an external standard containing 5 ng/ml of B_1 and G_1 and 1.5 ng/ml of B_2 and G_2. The limits of quantification were 0.5 ng of B_1 and G_1 and 0.15 ng of B_2 and G_2 per milliliter of culture medium.

Competition experiments. For each of the four VCGs, a *w afl*$^-$ mutant was combined with a *y* mutant of the same isolate, a *y* mutant of a different isolate within the same VCG, and three *y* mutants from different VCGs. Conidia from each mutant were obtained from 10 Cz slant cultures grown for 14 days at 30°C. After suspending in water with Tween 20 and filtering through glass wool, conidia were counted with a hemacytometer (clusters of two or more conidia were counted as single units) and adjusted to 10^6/ml with distilled water. The conidial suspension was diluted and plated (0.2 ml/plate) to give an estimated 100 colonies on each

of 10 plates of mYES with 2% agar (36 h at 30°C). The final conidial suspension was adjusted to yield 10^5 colonies/ml.

Each *w afl*$^-$ mutant was paired with *y* mutants by mixing the conidial suspensions to give estimated proportions of 0, 1, 10, 50, and 100% *w afl*$^-$ colonies upon plating. Conidial suspensions of mutants were combined in test tubes to a final volume of 5 ml; therefore, all proportions yielded 10^5 total colonies/ml. Conidial mixtures were warmed to 48°C in a water bath, and 1.25 ml was added to 100 ml of molten mYES agar precooled to 48°C. After thoroughly stirring the medium, 8 ml was pipetted into each of six replicate 60 × 15-mm plates, which gave 10^4 total colonies/plate. The four *w afl*$^-$ mutants were plated with *y* mutants on separate days. Plates were incubated in darkness for 5 days at 30°C.

Quantifying heterokaryosis, sporulation, and sclerotium production. Three of the six replicate plates from each treatment were examined for heterokaryons. These were recognized by a mixture of conidial chains consisting of green and one or both parental colors (white and yellow), all arising from a single conidiogenous structure (Papa, 1978). Conidiogenous structures with mixed conidial chains (heterokaryotic heads) were counted with a stereomicroscope equipped with an ocular grid at magnifications that gave areas of measurement ranging from 4 to 14.5 mm^2, depending upon the density of the heads. Heterokaryotic heads within three randomly chosen areas of each plate were counted; the final number of heads for each of three areas was calculated for 1 cm^2.

After examining the three plates for heterokaryotic heads, conidia from the plates were dilution-plated to indirectly measure colonization of the agar medium by *w afl*$^-$ and *y* mutants. The entire contents of each plate were added to 200 ml of distilled water with Tween 20 in a 500-ml Erlenmeyer flask and shaken 30 times by hand. Five milliliters of the conidial suspension was removed and diluted with distilled water, and 0.2 ml of the dilution was spread on each of three replicate plates (five plates for the time course; Fig. 1) of mYES agar with added 4 mg/liter dichloran (2,6-dichloro-4-nitroaniline; dissolved in acetone) to restrict colony diameter. Plates were incubated for 3 days at 30°C. Final percentages of *w afl*$^-$ mutants were based on proportions of *w afl*$^-$ and *y* colonies on dilution plates. For colonies consisting of a mixture of conidial colors, it was not possible to distinguish colonies that arose from heterokaryotic conidia and those resulting from overlapping colonies; therefore, all mixed colonies were counted as both *w afl*$^-$ and *y* mutants. Percentages of *w afl*$^-$ mutants were corrected for differences in sporulation between *w afl*$^-$ and *y* mutants, as determined from dilution plates with 100% *w afl*$^-$ and 100% *y* mutants.

Sclerotia were collected by autoclaving the above mixtures of conidia and agar medium and filtering through filter paper (Whatman No. 4; diameter, 5.5 cm). Filter papers were dried at 60°C for 5 h. Following removal from the filter paper, sclerotia were stored in a desiccator before weighing.

Aflatoxin analysis of mYES plates. The three remaining replicate plates from each treatment were frozen and analyzed for aflatoxins within 4 weeks. The contents of each plate were ground with a mortar and pestle in 7.5 ml of chloroform. The extract was transferred to a test tube along with the plate contents, and 7.5 ml of additional chloroform used to rinse the mortar and pestle was added to the test tube. The 15 ml of extract and the plate contents were vortexed for 30 s and filtered through a glass fiber filter. Final extracts were first analyzed by thin-layer chromatography (TLC) to determine the presence of aflatoxins so that appropriate dilutions could be made prior to quantification by HPLC. Two microliters of each extract was spotted along with a standard mixture of aflatoxins B_1, B_2, G_1, and G_2 on 10 × 10-cm silica gel 60 F-254 HPTLC plates and developed in a solvent system of chloroform–acetone (93:7, v/v). Aflatoxins were detected as blue fluorescent spots, and extracts were diluted as necessary based on the intensity of the fluorescence. If it was determined that no dilution was required prior to HPLC analysis, 1.5 ml of extract was evaporated to dryness under a stream of nitrogen and redissolved in 3.2 ml of HPLC injection solvent (methanol–water–acetic acid, 62:38:0.1, v/v/v). HPLC quantification was carried out as described earlier for vial cultures.

Statistics. Data were analyzed using SigmaStat version 1.0 (Jandel Scientific, San Rafael, CA).

RESULTS

Pairs of wild-type isolates within a VCG showed significant differences in one or more characters that included aflatoxin B_1 production, kojic acid production, number of sclerotia per plate, and sclerotium shape as measured by length/width ratio (Table 1). An exception was VCG 5, in which the two isolates did not differ in any of the characters examined.

Mutants *w afl*$^-$ and *y* were obtained from wild-type isolates. Heterokaryotic heads, recognized by a mixture of white and green, yellow and green, or white, yellow, and green chains of conidia, were detected in all pairings of mutants from the same isolate (Table 2). In addition, heterokaryotic heads were present in pairings of different isolates from the same VCG, with the exception of VCG 9, in which heterokaryotic heads were not detected in P23 *w afl*$^-$ + P53 *y* despite the formation of heterokaryons using complementary nitrate-nonutilizing mutants (Horn and Greene, 1995). In the pairing P12 *w afl*$^-$ + P48 *y*, sporulation by P48 *y* was sparse and nearly all heads with yellow-conidial

TABLE 1

Phenotypic Characters of Wild-Type *A. parasiticus* Isolates within Vegetative Compatibility Groups[a]

VCG	Isolate	Aflatoxin B_1 (μg/ml)[b]	Kojic acid (μg/ml)[b]	No. sclerotia/plate[c]	Sclerotium length/width ratio[d]
2	P5	120 ± 10.0**	99 ± 2.5 NS	213 ± 39.5***	1.3 ± 0.17 NS
	P43	156 ± 1.3	102 ± 5.7	3 ± 4.9	1.4 ± 0.26
3	P19	82 ± 5.6 NS	44 ± 3.2 NS	500 ± 29.8****	1.1 ± 0.08****
	P52	91 ± 7.5	44 ± 5.6	4 ± 3.0	1.3 ± 0.12
5	P12	167 ± 30.8 NS	137 ± 1.6 NS	0 ± 0.0 NS	—[e]
	P48	177 ± 12.0	128 ± 16.9	0 ± 0.0	—
9	P23	97 ± 7.0*	99 ± 5.5**	3 ± 2.3 NS	1.4 ± 0.21
	P53	137 ± 17.1	127 ± 6.9	0 ± 0.0	—

[a] Mycotoxin and sclerotium characters were determined according to the methods of Horn *et al.* (1996). Within each VCG, characters of the two isolates were compared with the *t* test. Probabilities: NS, not significant (>0.05); *, ≤0.05; **, ≤0.01; ***, ≤0.001; ****, ≤0.0001.
[b] Mean ± SD based on three replicate vial cultures.
[c] Mean ± SD based on three replicate plates.
[d] Mean ± SD based on 10–30 sclerotia from three replicate plates (10 sclerotia/plate, if available).
[e] Sclerotia not produced.

chains were heterokaryotic. The highest number of heterokaryotic heads occurred in proportions closest to a 1:1 ratio of *w afl*$^-$ to *y* mutants as determined from plate colonies (dilution-plating of conidia from colonies on experimental plates) (Table 2). Heterokaryotic heads were not observed in any pairings of mutants from different VCGs (data not shown). In most isolates, *y* mutants showed a reduced growth rate compared to *w afl*$^-$ mutants. This was reflected by a higher percentage of *w afl*$^-$ mutants and a corresponding lower percentage of *y* mutants when based on the colonization of the experimental plates instead of the original conidial inoculum (Table 2).

When cultured alone, P5 *w afl*$^-$ and P12 *w afl*$^-$ mutants did not produce sclerotia on mYES plates. In contrast, P19 *w afl*$^-$ produced 1281 ± 106.8 sclerotia/plate (n = 3; ±SD) weighing 48.1 ± 0.84 mg, and P23 *w afl*$^-$ produced 541 ± 17.6 sclerotia/plate weighing 11.7 ± 0.54 mg. None of the *y* mutants produced sclerotia, with the exception of P52 *y*, which produced 21 ± 13.9 sclerotia/plate weighing 2.7 ± 1.73 mg. Sclerotium production (number and dry weight) was examined when P19 *w afl*$^-$ and P23 *w afl*$^-$ were paired with *y* mutants on mYES plates that were inoculated with a conidial suspension comprising 50% *w afl*$^-$ mutant (Table 3). When P19 *w afl*$^-$ was combined with P19 *y* and P52 *y* from the same VCG, a significantly higher number and dry weight of sclerotia were produced than those of combinations involving different VCGs. Similarly, P23 *w afl*$^-$ paired with P23 *y* from the same isolate produced a significantly higher number and dry weight of sclerotia, but not when paired with a different isolate (P53 *y*) from the same VCG in which heterokaryotic heads were not present.

Maximum levels of aflatoxin B_1 in pairings of *w afl*$^-$ and *y* mutants on mYES plates occurred after 3 days of incubation and changed little at 5 days, when hetero-

TABLE 2

Heterokaryosis in Pairings of *A. parasiticus* Mutants from the Same Isolate and from Different Isolates within the Same Vegetative Compatibility Group

VCG	Mutant combination[a]	% *w afl*$^-$ mutant in conidial inoculum	% *w afl*$^-$ mutant as plate colonies[b]	No. heterokaryotic heads/cm^2[c]
2	P5 *w afl*$^-$ + P5 *y*	1	14.2 ± 6.32	113 ± 16.2
		10	46.6 ± 0.55	175 ± 29.4
		50	90.1 ± 4.42	28 ± 9.7
	+ P43 *y*	1	11.5 ± 1.93	68 ± 8.6
		10	47.7 ± 11.00	74 ± 28.7
		50	87.1 ± 7.69	38 ± 14.1
3	P19 *w afl*$^-$ + P19 *y*	1	79.1 ± 0.42	68 ± 22.5
		10	95.3 ± 1.31	11 ± 1.6
		50	99.6 ± 0.23	3 ± 1.3
	+ P52 *y*	1	82.2 ± 2.40	94 ± 7.3
		10	97.4 ± 0.11	18 ± 6.0
		50	99.7 ± 0.08	3 ± 2.1
5	P12 *w afl*$^-$ + P12 *y*	1	64.6 ± 5.76	29 ± 11.6
		10	89.2 ± 1.11	18 ± 8.6
		50	98.7 ± 0.81	1 ± 1.0
	+ P48 *y*	1	98.7 ± 0.52	123 ± 16.7
		10	99.7 ± 0.07	58 ± 6.7
		50	99.9 ± 0.09	5 ± 4.0
9	P23 *w afl*$^-$ + P23 *y*	1	54.3 ± 0.31	90 ± 23.7
		10	89.3 ± 0.52	36 ± 13.1
		50	98.7 ± 0.24	2 ± 1.5
	+ P53 *y*	1	37.2 ± 4.87	0 ± 0.0
		10	70.5 ± 5.05	0 ± 0.0
		50	93.7 ± 1.88	0 ± 0.0

[a] Mutation abbreviations: *w afl*$^-$, white conidia and does not produce aflatoxins; *y*, yellow conidia (produces aflatoxins).
[b] Mean ± SD based on three replicate plates. Percentages were determined from dilution-plating of conidia from colonies on experimental plates.
[c] Mean ± SD based on the number of heterokaryotic heads on the surface of three replicate plates.

karyotic heads were detectable and plates were analyzed for aflatoxins (Fig. 1). Increasing percentages of *w afl*$^-$ mutants with concomitant decreases in the number of aflatoxigenic *y* colonies on plates resulted in decreasing concentrations of aflatoxin B_1 (Fig. 2). Strains of *w afl*$^-$ differed in their capacity to inhibit aflatoxin production by *y* mutants; for example, P12 *w afl*$^-$ was ineffective at reducing aflatoxin B_1 except at high levels of plate colonization. There were no consistent differences in aflatoxin B_1 inhibition by *w afl*$^-$ mutants in pairings of mutants from the same VCG (either the same or a different isolate) and pairings of mutants from different VCGs.

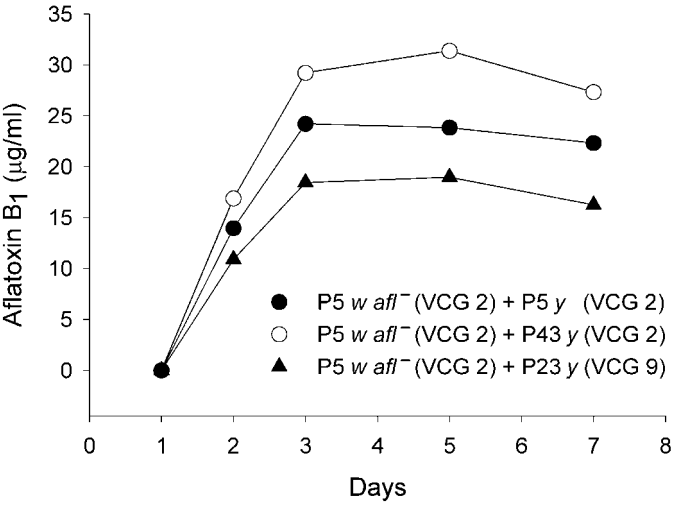

FIG. 1. Production of aflatoxin B_1 by *A. parasiticus* on mYES plates at different incubation periods at 30°C. A nonaflatoxigenic white-conidial mutant (P5 *w afl*$^-$) was paired with aflatoxigenic yellow-conidial mutants from the same isolate (P5 *y*), from a different isolate within the same VCG (P43 *y*), and from an isolate belonging to a different VCG (P23 *y*). Percentages of P5 *w afl*$^-$ (mean ± SD; $n = 5$) at 5 days, determined from dilution-plating of conidia from colonies on experimental plates originally inoculated with a conidial suspension comprising 50% P5 *w afl*$^-$, were 49.3 ± 3.60% (+P5 *y*), 52.2 ± 4.0% (+P43 *y*), and 44.5 ± 7.7% (+P23 *y*). Numbers of heterokaryotic heads/cm^2 on the plate surface (mean ± SD; $n = 3$) at 5 days were 365 ± 14.7 (+P5 *y*), 344 ± 15.2 (+P43 *y*), and 0 ± 0.0 (+P23 *y*).

DISCUSSION

In *A. parasiticus*, gene action determining conidial color is autonomous, meaning that conidial color is determined by the nuclei present in the conidium rather than by the nuclei within the conidiophore (nonautonomous) (Papa, 1978). Conidia of *A. parasiticus* typically contain three to four nuclei (Yuill, 1950), and complementation resulting in wild-type green color occurs when nuclei from both white- and yellow-conidial mutants are present in a single conidium. Ishitani and Sakaguchi (1956) showed that in *A. sojae*, considered a domesticated form of *A. parasiticus* used in food fermentations (Kurtzman *et al.*, 1986), the frequency of hyphal anastomosis between conidial-color mutants was positively correlated with the number of heterokaryotic heads. Anastomoses between compatible strains of *A. sojae* occurred primarily between conidia in very close proximity and involved a very short or no hyphal connection. Furthermore, the number of heterokaryotic heads in culture was highest under nutrient-deficient conditions with a high C/N ratio (Ishitani and Sakaguchi, 1956). In the present study, heterokaryon formation was encouraged by inoculation with a high density of conidia, resulting in 10^4 colonies per plate, and by a medium that contained suboptimal concentrations of nutrients for growth yet permitted aflatoxin B_1 production.

Heterokarysis, a prerequisite to genetic recombination through the parasexual cycle, occurred in this study only between vegetatively compatible strains. The parasexual cycle in *A. parasiticus* and *A. flavus* has been detected under laboratory culture conditions (Papa, 1973, 1978) but not in nature. Geiser *et al.* (1998) examined DNA sequences from five genes in *A. flavus* and found genetic variation consistent with a history of recombination, although the method of recombination, either through sexuality or through parasexuality, was not determined. They further speculated that recombination may be possible in *A. flavus* between an intro-

TABLE 3

Sclerotium Production by *A. parasiticus* in Pairings of Mutants from the Same and Different Vegetative Compatibility Groups

Mutant combination[a]	% *w afl*$^-$ mutant as plate colonies[b]	Heterokaryotic heads[c]	No. sclerotia (dry wt., mg) per plate[d]	
P19 *w afl*$^-$ (VCG 3)				
+ P52 *y* (VCG 3)	82.2 ± 2.40	+	822 (39.5)	A
+ P19 *y* (VCG 3)	79.1 ± 0.42	+	746 (36.1)	A
+ P48 *y* (VCG 5)	63.4 ± 7.93	−	329 (14.8)	B
+ P23 *y* (VCG 9)	85.1 ± 1.91	−	319 (14.0)	B
+ P43 *y* (VCG 2)	79.3 ± 0.88	−	180 (7.6)	C
P23 *w afl*$^-$ (VCG 9)				
+ P23 *y* (VCG 9)	54.3 ± 0.31	+	289 (4.6)	A
+ P43 *y* (VCG 2)	43.6 ± 5.56	−	29 (0.5)	B
+ P48 *y* (VCG 5)	54.1 ± 2.70	−	23 (0.5)	B
+ P19 *y* (VCG 3)	69.0 ± 4.68	−	17 (0.3)	B
+ P53 *y* (VCG 9)	37.2 ± 4.87	−	4 (0.06)	B

[a] Mutation abbreviations: *w afl*$^-$, white conidia and does not produce aflatoxins; *y*, yellow conidia (produces aflatoxins).

[b] All mutant pairings based on plates ($n = 3$) inoculated with a conidial suspension comprising 50% *w afl*$^-$ mutant; final percentages shown (mean ± SD) were determined from dilution-plating of conidia from colonies on experimental plates.

[c] Numbers of heterokaryotic heads indicated in Table 2.

[d] Means based on three replicate plates. For each set of five pairings with a *w afl*$^-$ mutant, means of both sclerotium number and dry weight not sharing a common letter are significantly different ($P \leq 0.05$) based on an ANOVA followed by Student–Newman–Keuls test for comparison of means.

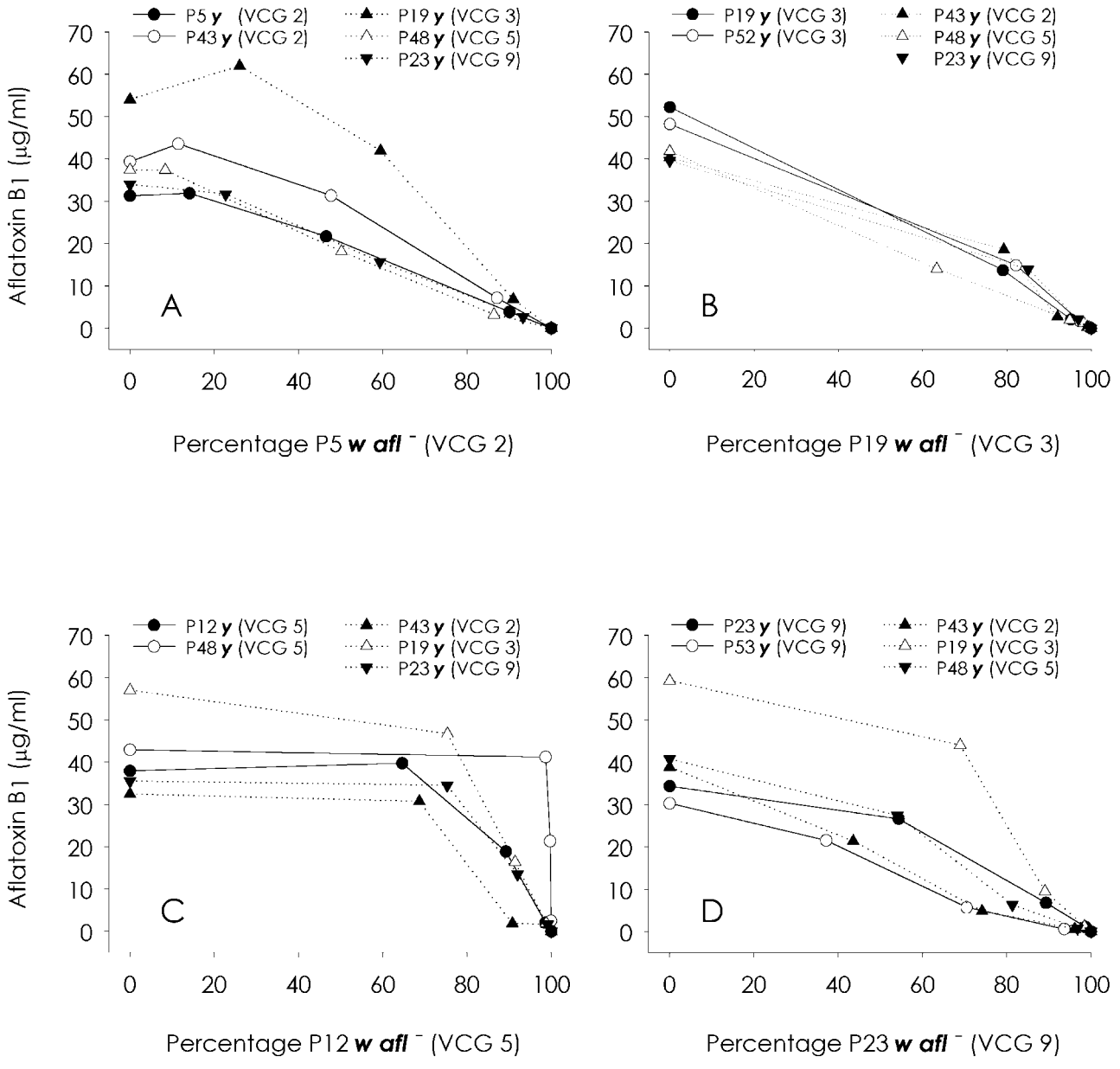

FIG. 2. Inhibition of aflatoxin B_1 production by *A. parasiticus* on mYES plates when paired with different percentages of nonaflatoxigenic white-conidial mutants (*w afl*$^-$). Percentages of *w afl*$^-$ mutants were determined from dilution-plating of conidia from colonies on experimental plates originally inoculated with a conidial suspension comprising 0, 1, 10, 50, and 100% *w afl*$^-$ mutants. Numbers of heterokaryotic heads/cm^2 on the plate surface are indicated in Table 2. (A) P5 *w afl*$^-$ paired with aflatoxigenic yellow-conidial mutants from the same isolate (P5 *y*), from a different isolate within the same VCG (P43 *y*), and from isolates belonging to different VCGs (P19 *y*, P48 *y*, and P23 *y*). (B) P19 *w afl*$^-$ paired with aflatoxigenic yellow-conidial mutants from the same isolate (P19 *y*), from a different isolate within the same VCG (P52 *y*), and from isolates belonging to different VCGs (P43 *y*, P48 *y*, and P23 *y*). (C) P12 *w afl*$^-$ paired with aflatoxigenic yellow-conidial mutants from the same isolate (P12 *y*), from a different isolate within the same VCG (P48 *y*), and from isolates belonging to different VCGs (P43 *y*, P19 *y*, and P23 *y*). (D) P23 *w afl*$^-$ paired with aflatoxigenic yellow-conidial mutants from the same isolate (P23 *y*), from a different isolate within the same VCG (P53 *y*), and from isolates belonging to different VCGs (P43 *y*, P19 *y*, and P48 *y*).

duced nonaflatoxigenic biological control strain and native aflatoxigenic strains in the field. Recombination through the parasexual cycle would occur between vegetatively compatible strains that differ in genotype, and rare instances of such recombination would not likely influence the immediate effectiveness of a biocontrol strain. However, the creation of new genotypes in which an aggressive biocontrol strain becomes aflatoxigenic is a concern that can be addressed only through the use of sensitive molecular genetic techniques on field populations.

Hyphal anastomoses between vegetatively compat-

ible strains of *A. parasiticus*, as detected by the presence of heterokaryotic heads, were associated with higher sclerotium production than that in pairings of strains from different VCGs in which there was no evidence of heterokaryosis (Table 3). These data support the postulate that when strains from the same VCG are combined, the mycelium derived from hyphal anastomoses has a larger substrate base for producing sclerotia than that of more restricted colonies resulting from competition between different VCGs (Wicklow, 1990). Garber and Cotty (1997) reported that when isolates of *A. flavus* that were presumably from different VCGs were coinoculated into developing cotton bolls, sclerotium formation on cottonseed was greatly inhibited. Similarly, when canola stems were inoculated with mixtures of different genotypes of *Sclerotinia sclerotiorum* (Lib.) de Bary, sclerotial mass was usually reduced for each of the competing genotypes (Maltby and Mihail, 1997). Hence, sclerotium production resulting from competition in *A. parasiticus* between a biological control strain and a native strain on a crop plant may depend on whether the strains are vegetatively compatible.

The higher sclerotium production associated with pairings of vegetatively compatible strains suggested that combining nonaflatoxigenic mutants with compatible aflatoxigenic strains would result in more aflatoxin B_1 than combining strains that are incompatible. Hyphal anastomoses with the *w afl*$^-$ mutant could create a larger substrate base for aflatoxin production by the *y* mutant; alternatively, the aflatoxigenic *y* mutant could provide missing aflatoxin pathway compounds for the *w afl*$^-$ mutant. However, the presence of heterokaryotic heads in *A. parasiticus* was not associated with higher aflatoxin B_1 production than that of mixtures of strains from different VCGs. This suggests that there was no effect of hyphal anastomosis on aflatoxin production, or that the effect was localized at the point of anastomosis and was not detectable by the analyses used in this study. Furthermore, there was no indication that inhibition of aflatoxin B_1 production was due to factors other than the competition for nutrients, an interaction termed exploitation competition (Lockwood, 1992). At a 1:1 mixture (50% *w afl*$^-$ mutant determined from sporulation on experimental plates) of nonaflatoxigenic and aflatoxigenic colonies, reduction of aflatoxin B_1 rarely exceeded 50% (Fig. 2). These results differ from the study of Ehrlich (1987) in which wild-type *A. parasiticus* was paired with mutants from the same isolate blocked at different steps along the aflatoxin pathway. A 6- to 21-fold reduction in aflatoxin B_1 was reported for strains in a 1:1 mixture (also based on the amount of sporulation in culture). Substantial inhibition of aflatoxin B_1 production also was reported when nonaflatoxigenic and aflatoxigenic *A. flavus* isolates from different VCGs were cultured together (Cotty and Bayman, 1993). The experiments of both Ehrlich (1987) and Cotty and Bayman (1993), which involved cultivation of fungi in liquid culture, suggest that inhibition of aflatoxin under these conditions involves factors other than competition for nutrients by the nonaflatoxigenic strain. The apparent absence of such factors in this study is not easily explained, but differences in cultural conditions, such as the use of an agar medium, may be responsible.

VCGs of *A. flavus* and *A. parasiticus* are distributed over large geographic regions of the United States (Horn and Dorner, 1998, 1999). In addition, diversity of *A. flavus* and *A. parasiticus* VCGs in populations within a single field is high (Bayman and Cotty, 1991; Horn and Greene, 1995), which suggests that competition during seed invasion between native strains and an applied nonaflatoxigenic strain of either species will likely involve different VCGs. Since data presented here showed no difference in inhibition of aflatoxin B_1 production according to whether competing strains are vegetatively compatible, the VCG composition of native strains within a field may be of minor importance in predicting the efficacy of a particular nonaflatoxigenic biocontrol strain. Other strain characteristics affecting competitive ability, such as enzyme production, growth rate, and capacity to survive in soil, may be more important in determining the success of a biocontrol strain in inhibiting aflatoxin contamination within a crop.

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